ISSN 2278-7763

Characterization of Trypsin Inhibitor from the seeds of *Achyranthes aspera*

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ABSTRACT

Achyranthes aspera seeds trypsin inhibitor was found to be rich in acidic amino acids, serine and it was devoid of free thiol groups and tryptophan . The inhibitor was stable under conditions of extremes of pH (3·0–12·0), at high temperatures and in the presence of denaturing agents. AATI was a glycoprotein with a carbohydraye content of 2.5%. The inhibitor showed a non-competitive type of inhibition with K_i value of 2.9×10^{-10}M for bovine trypsin**.** AATI formed a stable complex with trypsin in a 1:1 molar ratio. AATI. Chemical modification of lysine residues of the inhibitor resulted in the loss of its trypsin inhibitory activity indicating that amino groups are essential for its activity.

Key words: *Achyranthes aspera seeds*, Trypsin inhibitor, trypsin- trypsin inhibitor interactions, kunitz family.

INTRODUCTION

Protease inhibitors active against proteolytic enzymes are widely distributed in nature. These antinutritional factors have been isolated and characterized from a number of plant and animal tissues. The mode of action of protease inhibitors is now well established.(1) Functionally, these proteins are concerned with the regulation of endogenous protease activities and supplying amino acids as storage proteins apart from protecting plant tissues from pest and pathogen attack. The present work deals with the studies related to amino acid composition, physiochemical properties , mode of action and specifity of trypsin inhibitor.

MATERIAL AND METHODS

Materials:

Achyranthes aspera trypsin (AATI) inhibitor sample was isolated from the seeds of prickly chaff flower by the procedure described in an earlier paper (Geetha and Siva Prasad 2013).

Chemicals and Enzymes

Bovine pancreatic trypsin (1 x crystallized, DCC-treated, type xi), bovine pancreatic α-chymotrypsin (3 x crystallized, type ii), molecular markers, soybean trypsin inhibitor (type I-S), soybean Bowman-Birk inhibitor, N-acetyl-DL-phenylalanyl-β-naphthylester (APNE), N-acetyl-L-tyrosine ethyl ester (ATEE), α-N-benzoyl-DL-arginine-p-nitoanilide HCl (BAPNA), blue dextran, DEAE-cellulose, N,N-dimethylformamide, N,N-dimethylsulfoxide, N,N'-methylene bis acrylamide, sodium dodecyl sulfate, diazoblue-B were purchased from Sigma Chemical company, St. Louis, Missouri, U.S.A. Sephadex G-100 and Sephadex G-200 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Acrylamide was purchased from J.T. Baker Chemical Company, Phillipsburg, N.J., U.S.A.

ISSN 2278-7763

N,N,N',N' – tetramethylene 1,2 diaminoethane (TEMED) was purchased from B.D.H. Chemical Ltd., Poole, England. Coomassie brilliant blue R250 was from Kochlight Laboratories Ltd. Colnbrook, Berks, England. All other chemicals used were of analytical grade.

Methods

Protein estimation.

Protein was estimated by the method of Lowry *et al*.(2) using BSA as the standard. The protein content in the column effluents collected during chromatographic separation were determined by measuring the absorbance at 280 nm.

Amino acid analysis

Hydrolysis of protein: 5 mg of AATI was taken in a test tube and 1 ml of 6 N HCl was added to it. The tube was sealed under nitrogen atmosphere and the protein was hydrolysed at 110°C according to the method of Moore and Stein (3). Amino acids were measured by Ion exchange chromatography using Ninhydrin post column derivatization through amino acid analyser.

Determination of tryptophan . Tryptophan content of the inhibitor was determined by the spectrophotometric method of Edelhoch (4).

Estimation of carbohydrate content. Neutral sugar content of AATI was determined by the method of Dubois *et al*.(5) using D-mannose as the standard.

Estimation of thiol groups. The free thiol groups in the inhibitor were estimated according to the procedures of Ellman (6) and Habeeb (7) using 5,5´dithiobis-(2-nitrobenzoic acid) (DTNB).

Stability of the inhibitor under extreme conditions

Effect of pH and temperature. Solutions of the inhibitor (1 mg/ ml) in 10 mM buffers of five pH values (pH 3.0, glycine-HCl; pH 5.0, sodium citrate; pH 7.0, sodium phosphate; pH 9.0, Tris-HCl; pH 12.0, glycine-NaOH) were kept at 5°C for 24 h. Aliquots of the inhibitor were diluted with 0.1M phosphate buffer, pH 7.6 and assayed for trypsin inhibitory activity. For studying the effect of temperature ,the inhibitor solutions (100 µg/ml) were separately incubated in a water bath at various temperatures for 10 min and then quickly cooled in ice, and appropriate aliquots were assayed for TIA.

Effect of denaturants. AATI **(**1 mg / ml) in 0.1 M phosphate buffer, pH 7.6 containing 8 M urea was incubated at 5°C for 24 h and assayed for TIA.To determine the stability of the inhibitor in 6M guanidine hydrochloride and 10g/l SDS,1 mg/ ml solution of AATI in 0.1 M phosphate buffer, pH 7.6 containing 6M guanidine hydrochloride (GuHCl)or 10g/l SDS, were kept at room temperature for 24 h and dialysed against the buffer for 12h and used for assaying antitryptic activity.

Specificity /Enzyme assay methods. The inhibition spectrum of AATI was established by first assaying the proteinase or esterase activity of the enzyme on an appropriate substrate and then incubating a fixed amount of the enzyme with various amounts of the inhibitor and assaying the residual enzyme activity.The activities of trypsin and pronase or their inhibition were assayed by the method of Kakade *et al* (8) using either BAPNA or casein as the substrate. The inhibitory activity towards chymotrypsin was determined using ATEE as the substrate (9) . The proteolytic activity of papain was assayed using casein as the substrate by the method of Arnon (10). The esterolytic activity of subtilisin was assayed using ATEE (11) as the substrate. Thermolysin was assayed according to the method of Matsubara (12). Pancreatic α-amylase was assayed by the method of Saunders and Lang (13) using starch as the substrate.

ISSN 2278-7763

Kinetic studies. The amidolytic activity of trypsin (30 µg) was determined with various concentrations of BAPNA(0.8 to 5.0 µmol) in the absence of the inhibitor. The assays were repeated in the presence of 10µg, 15µg, 20 µg of the inhibitor. Ki value of trypsin was calculated from Lineweaver-Burk plots.

Formation of Enzyme- Inhibitor complex and its Molecular weight determination. The trypsin-inhibitor complex was isolated by gel filtration on Sephadex G-200. For the isolation of the trypsin-inhibitor complex, a mixture of the inhibitor (2 mg) and trypsin (5 mg) in 1 ml of 0.1 M phosphate buffer, pH 7.6, was allowed to stand at room temperature for 15 min and then chromatographed. The absorbance of fractions (1.5 ml) was measured at 280 nm and the trypsin inhibitory activity in the fractions was determined. The molecular weight of trypsin-AATI complex was determined by the method of Andrews (14) using standard protein markers for calibration.

Chemical modification of the inhibitor. Modification of amino groups using acetic anhydride was done according to the method of Rice and Etzler (15). Progressively larger quantities of acetic anhydride in the range 1-20 µl were added to a series of AATI solutions (2 mg in 5 ml of 0.3 M N-ethylmorpholine acetate buffer, pH 8.5). Each mixture was dialysed against double distilled water for 48 h and assayed for residual inhibitory activity. The extent of acetylation was determined using TNBS by the method of Haynes *et al.* (16). To 1ml of the acetylated inhibitor solution, 1 mL of 5% $NaHCO_3$, pH 8.5, and 1ml of 0.1% TNBS in water was added. The mixture was incubated at 40°C for 2 h. The reaction was arrested by adding 1 ml of 10% SDS solution and 0.5 ml of 1 N HCl. The absorbance of the solution was read at 344 nm against a blank treated as above but containing 1 ml of water instead of protein solution. TIA of the modified inhibitor was determined.

Arginyl residues were modified with 1, 2-cyclohexanedione (CDH) according to the method of Liu *et al*. (17).To the inhibitor (1mg in 4.5 ml of 1mM triethanolamine buffer, pH 8.0), CDH (1 mM and 5 mM) in 0.5 ml of water was added. In the control, water without CDH was added.The reaction was allowed to proceed at room temperature in the dark for 12 h.The mixture was then dialysed against double distilled water for 48 h, lyophilized and assayed for TIA.

RESULTS

Determination of aminoacid content. The results of the amino acid analysis is shown in the **Table1.**The amino acid composition showed that the inhibitor contained all the amino acids that are normally found in proteins. The inhibitor contained smaller amounts of half cystine, methioninie, tyrosine, phenylalanine, histidine, isoleucine, leucine and arginine. It was rich in acidic amino acids, serine, alanine, lysine, glycine, threonine, valine which together accounted for more than 75% of the total amino acid content. Characteristic features of the amino acid composition of AATI is that it contained low half cystine and it is free from tryptophan and proline residues.The inhibitor was found to be glycoprotein with a neutral sugar content of 2.5%. It was devoid of amino sugars and free sulfhydryl groups.

Effect of pH ,temperature and denaturants on the stability of the inhibitor. AATI was preincubated with buffers of different pH in the range of 3 to 12 and subsequently assayed for the trypsin inhibitory activity at pH 7.6.**(Table 2)** shows that trypsin inhibitory activity of AATI was not affected at alkaline as well as in acidic condition. The purified inhibitor was quite stable up to 90°C for 10 min. When the incubation

ISSN 2278-7763

was prolonged for 20 min, there was 30% loss of its activity. But when kept in boiling water bath, there was 40% loss of activity in 10 min and incubation of AATI for 30 min at boiling temperature caused the total loss of trypsin inhibitory activity. Incubation with 8M urea or 10 g/l SDS for 24 h at room temperature did not affect the TIA of AATI. But contact with 6 M GuHCl for 24 h resulted in loss of 40% of its inhibitory activity. **(Table 3)**

Activity of AATI on other proteases. Of the several proteases tested, trypsin was found to be strongly inhibited by AATI, followed by elastase and *Staphylococcus aureus* protease with IC_{50} values 10, 20.7and 332.77 respectively. AATI did not inhibit the activities of papain, pepsin, pronase,thermolysin, subtilisin proteinase K and *Aspergillus oryzae* proteinase and α – amylase. From the results obtained, it is evident that AATI specifically inhibited some serine proteinases of mammalian and bacterial origin but not the enzymes of fungal origin. IC50 values for the inhibitor varied depending on the proteinase inhibited**.** **(Table 4)**

Table 1. Amino acid composition of AATI

Amino acid	Mole percent
Aspartic acid	16.13
Threonine	5.55
Serine	26.45
Glutamic acid	5.48
Proline	_
Glycine	5.59
Alanine	7.47
Cysteine	2.24
Valine	4.36
Methionine	2.78
Isoleucine	2.44
Leucine	3.66
Tyrosine	3.37
Phenylanine	3.08
Histidine	2.29
Lysine	6.81
Arginine	1.62
Tryptophan	_

Aspartic and glutamic acid values include aspargine and glutamine respectively.

Table 2. Effect of pH on AATI

pH	Name of the buffer	TIU/mgofAATI x 10^2
3	Glycine-HCL	43.1
5	Sodium citrate	43.5
7	Sodium Phosphate	44.2
9	Tris-HCL	44.1
12	Glycine-NaOH	43.6

AATI was incubated for 24 h at 4°C in the respective buffers and assayed for TIA using BAPNA as the substrate.

Table 3. Effect of 8M urea, 1% SDS and 6M GuHCl on AATI

Denaturants	TIU/mg of AATIx 10^2
Control	43.2
8M urea	42.5
1 % SDS	42.7
6M GuHCl	24.8

AATI was incubated for 24 h at room temperature (29°C) in 8 M urea, 1% SDS, 6 M GuHCl and assayed for TIA using BAPNA as the substrate.

Table 4. Activity of AATI on Proteinases

Enzyme	AATI	
	Inhibitory effect	IC50 * value
Trypsin	Positive (42.5 x 10^2 TIU)	10
Chymotrypsin	--	--
Elastase	Positive (16.4 x 10^2 EIU)	20.7
Pronase	--	--
Staphylocccus aureus Protease	Positive (4.4 x10^2 SAPIU)	332.77
Subtilisin BPN	--	--
Subtilisin Carlsberg	--	--
Proteinase K	--	--
Aspergillus oryzae protease	--	--
Thermolysin	--	--
Papain	--	--
Pepsin	--	--

-- means Inhibition not observed even at a concentration of 500 µg of AATI.*IC_{50} value represents µg of AATI required to cause 50% inhibition of the enzyme activity.

ISSN 2278-7763

Mode of inhibition of trypsin .Trypsin activity in the presence (10, 15, 20 µg inhibitor) and absence of AATI was measured at different substrate (0.8 to 5 µmol BAPNA) concentrations. The double reciprocal plot of the kinetic data is shown in Fig. 11A. In the presence of inhibitor, there was a decrease in the V_{max} and the curves met on the'Y' axis at a point equivalent to $-1/K_m$. The mode of inhibition of trypsin by AATI was non competitive. K_i value of trypsin for AATI, calculated from the Lineweaver-Burk plots, was 0.29 ± 0.07 nM. (**Fig 1)**

Interaction of AATI with inactive trypsinogen .In order to ascertain whether trypsinogen will interact with AATI and AATI-trypsin complex, experiments were carried out with varying amounts of trypsinogen in the assay system of AATI. It can be seen from Figure 6 that trypsin activity is not altered in the presence of trypsinogen. Similarly, trypsinogen did not have any effect on the inhibitory activity of AATI towards trypsin even when it was present at 5-6 fold weight excess. It is clear that a catalytically active enzyme is necessary for the formation of trypsin – inhibitor complex with AATI.(**Fig 2)**

Isolation of the trypsin-AATI complex. A mixture of 5mg of trypsin and 2mg of AATI incubated at room temperature for 15 min when applied on to a column of Sephadex G-200 at 5°C, previously calibrated with AATI (**Fig 3A)** and trypsin (**Fig 3B)** run separately, gave rise to 2 distinct A_{280} absorbing peaks (**Fig 3C**). The peak I had an elution volume (40 ml) which is lower than that of free AATI (64 ml). This fraction did not show any trypsin activity by itself nor was there any TIA associated with it. The molecular weight calculated for the trypsin-AATI complex in peak I, based on standard protein calibration curve gave a value of 45kDa. This would mean a mol/mol-interaction of the inhibitor of AATI with trypsin. Peak II showed trypsin activity but the specific activity appeared much less than the original trypsin activity. This is due to the excess trypsin added in the incubation medium. Similar results were also obtained when 1 mg of AATI was incubated with 3 mg of trypsin and the mixture was subjected to gel filtration on Sephadex G-200 column.

Chemical modification of AATI

Modification of arginyl residues with 1,2 – cyclohexanedione (CHD):

The effect of modification of arginyl residues of AATI with CHD on its trypsin inhibitory activity is shown in **Table 5.** There was a marginal loss (8%) in trypsin inhibitory activity on modifying arginine residues indicating that the arginyl residues in the inhibitor do not have any role in the inhibition of trypsin by AATI.

Acetylation of the inhibitor

AATI was treated with acetic anhydride in order to modify its free amino groups. The extent of acetylation of the inhibitor was determined along with its trypsin inhibitory activity.

Figure 4 shows loss in the TIA proportionate to the acetylation of AATI. 80% of the amino groups of the inhibitor could be acetylated even after using excess acetic anhydride resulting in the loss of about 80% of its trypsin inhibitory activity. The remaining amino groups, in the interior part of the molecule, may not be accessible to the chemical reagent. No attempt has been made to determine the number of amino groups modified. Loss of TIA of AATI on acetylation indicates that one or more amino groups of the inhibitor are needed for its inhibitory effect.

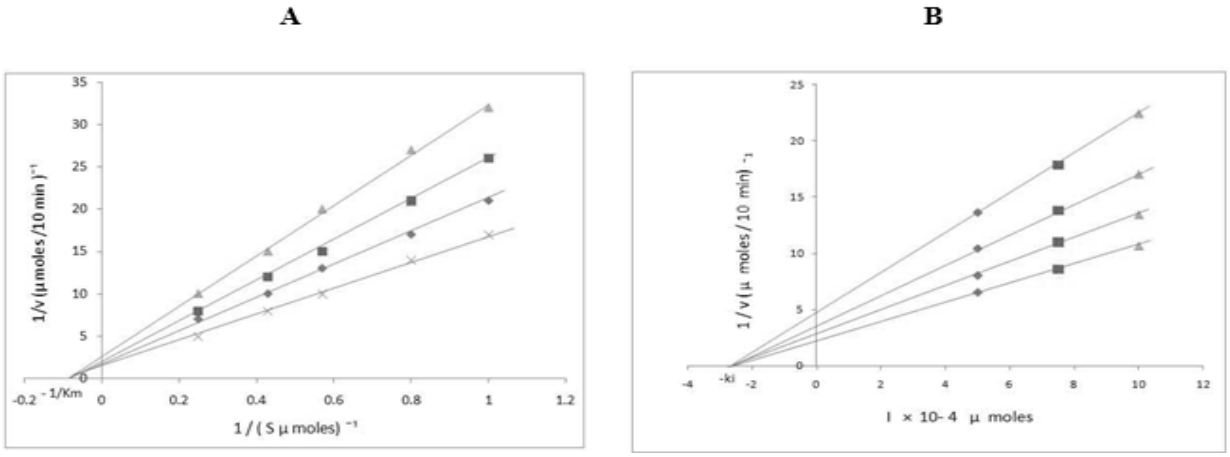

Fig1. Mode of inhibition of trypsin activity by AATI (**A**) Lineweaver-Burk plot **(B**) Dixon plot.Inhibition of amidolytic activity of trypsin by AATI was done by incubating 30 µg of trypsin and BAPNA solution (0.8 – 5 µmol) with the reaction system containing 10, 15 and 20µg of AATI. (- × -) Without AATI;(- ♦ -) 10 µg of AATI; (- ■ -) 15 µg of AATI; (-▲-) ; 20 µg of AATI

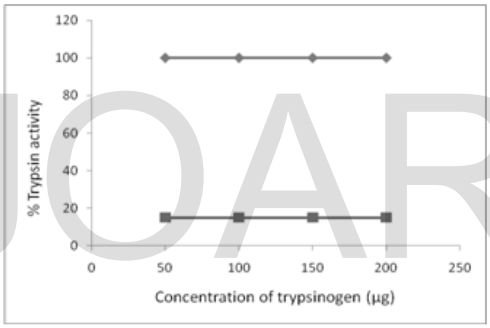

Fig2. 30µg of trypsin is treated with BAPNA in the presence of varying amounts of trypsinogen (♦) Trypsin activity b) Trypin inhibitory activity of AATI in the presence of varying amounts of trypsinogen (■) Trypsin - AATI complex

Table 5. Effect of modification of arginine on the trypsin inhibitory activity of AATI

Concentration of Cyclohexanedione	TIU/mg of inhibitor $\times 10^2$
Control	40.1
1 mM	39.1
5mM	36.8

Arginyl residues of AATI were chemically modified with 1, 2– cyclohexanedione. Trypsin inhibitory activity of the modified AATI was assayed using BAPNA as the substrate of trypsin.

ISSN 2278-7763

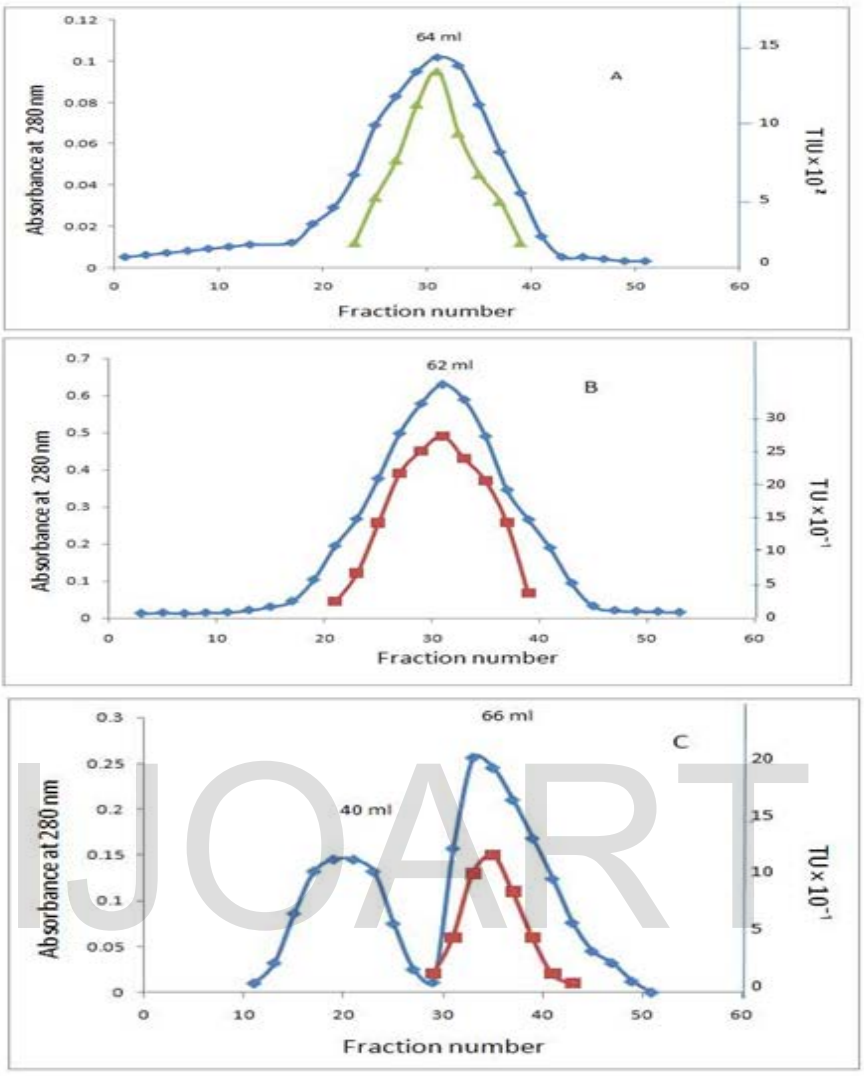

Fig 3. Elution patterns of AATI, trypsin , trypsin-AATI complex on Sephadex G-200 column (**A)** 2 mg AATI **(B)** 5 mg trypsin **(C)** 2 mg AATI+ 5 mg trypsin. 1.5 ml fractions were collected at a flow rate of 10ml/h Protein was monitored by measuring the absorbance at 280 nm (♦--♦); Trypsin activity (■--■); TIA (▲--▲)

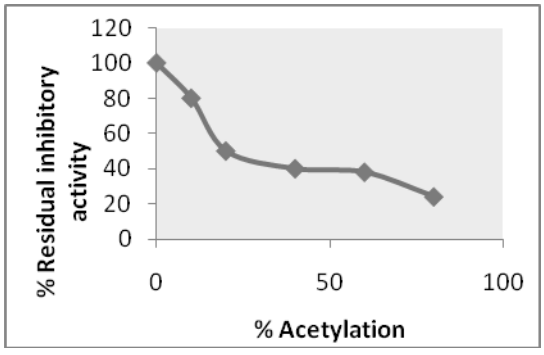

Fig4. Loss of inhibitory activity of AATI on acetylation with acetic anhydride

ISSN 2278-7763

DISCUSSION

Although AATI is a glycoprotein with 2.5% carbohydrate content, it is unlikely to show anomalous behavior on Sephadex beads since it moved out of them symmetrically with a narrow peak. Even though most of the protease inhibitors are non glycoproteins, some are reported to have carbohydrate moieties. Trypsin inhibitors from the seeds of *Swatzia pickellii*, *Echinodorus paniculatus*, *Peltophorum dubium*, *Carica papaya* are also reported to be glycoproteins (18-21).

Majority of plant protease inhibitors have molecular weights ranging from 8 to 20 kDa (22). A trypsin inhibitor from seeds of *Brassica campestris* (BCTI) had a molecular weight of 8 kDa (22). Most of the inhibitors isolated from legumes have molecular weights around 25 kDa. The amino acid composition of AATI is characterized by high content of acidic amino acids. This feature appears to be similar to the previously characterized protease inhibitors isolated from jack fruit seeds and also to some of the well characterized inhibitors from soybean (kunitz), lima bean, buckwheat inhibitor, cow pea trypsin inhibitors II and III, *Inga laurina* trypsin inhibitor,and sword bean trypsin inhibitor (23-28). AATI is unique in that it contained about 27% serine and with threonine, the hydroxy amino acids accounted for nearly one third of the total amino acids of the protein. AATI contained 4 half cystines and it was devoid of free thiol groups. The inhibitors isolated from soybean (Kunitz), navy bean, jack fruit seeds, cow pea, *Inga laurina*, sword bean (23,29,30,26-28) were reported to contain 2 to 6 half cystines residues with no free thiol groups. AATI contained no tryptophan residues similar to the protease inhibitors from chick pea (31), wheat germ (32), horse gram (33), ridge gourd (34) and cow pea (26).

The unusual stability of protease inhibitors, in general, is their most remarkable property. AATI showed similarities to protease inhibitors from a number of plant sources in their stability. (35-37). The low cysteine content in these inhibitors negates the possibility of the stability of the inhibitors rendered due to extensive intrapeptide crosslinking. However, the unusual stability of the inhibitor may be due to strong hydrophobic interactions forming an inner core in the protein or regeneration of normal active form after denaturation due to small size of the protein.The result of the investigation of the inhibitory specificity of AATI has shown it to be a serpin active against bovine trypsin, porcine elastase and *Staphylococcus aureus* protease. The inhibitor was ineffective against fungal enzyme tested.

The inhibitory activity of AATI was assessed by using different enzymes. AATI was not active against other proteases such as papain (thiol), pepsin (carboxyl) and thermolysin (metalloprotease). Majority of plant protease inhibitors isolated so far have been found to be specific for serine proteases (38). Some potent inhibitors of trypsin are inactive or only weakly active against chymotrypsin (39) and vice-versa (40). But in certain cases, they have been found to inhibit a range of other serine proteases such as elastase (41), thrombin (42), plasmin and kallikrein (43). AATI being active against trypsin and inactive towards chymotrypsin is similar to inhibitors isolated from the seeds of *Inga laurina* and *Spinacia oleracea* in its specificity (27,37). However, some serpins are reported to inhibit other classes of proteases. The trypsin/ chymotrypsin inhibitor from broad beans inhibited the sulfydryl enzyme papain (42). Serine protease inhibitors such as barley subtilisin inhibitor (44) and wheat germ protease K inhibitor (45) are found to be active against α – amylases.

ISSN 2278-7763

As regards the mechanism of action, AATI has shown non competitive type of inhibition. Although a few like, kunitz soybean trypsin inhibitor, *Archidendron ellipticum* inhibitor and *Entada scandens* inhibitor have shown competitive type of inhibition (23,36,28) majority of the inhibitors including those from *Artocarpus integrifolia*, faba bean, *Bauhinia bauhinioides* and *Inga laurina* followed non competitive inhibition kinetics. (30,46,47, 27). Jack fruit seed protease inhibitor isolated by Annapurna *et al.*(30) also showed non competitive enzyme inhibition but the one isolated by Bhat and Pattabiraman (48) exhibited uncompetitive inhibition. The K_i value of AATI was determined to be 2.9 $x10^{-10}$ M. Ki values reported for trypsin inhibitors from *Delonix regia*, *Peltophorum dubium*, *Spinacia oleracea* were 2.8 $x10^{-6}$ M, 2.19 $x10^{-8}$ M, 1.7 $x10^{-9}$ M, 4 $x10^{-10}$ M respectively and the one from *Peltophorum dubium* is close to the Ki value of AATI. (49,27,37). The low K_i value indicates high affinity of trypsin towards AATI.

When AATI was incubated with excess trypsin, a complex with molecular mass of 45kDa was observed by gel filteration on Sephadex G-200. The formation of stable trypsin-inhibitor complex suggests the binding of the inhibitor to trypsin in a 1:1 molar ratio without the formation of ternary complex. AATI is a mono-headed inhibitor with a site for trypsin.The stiochometric ratio of 1:1 and the molecular mass agreed with those for other kunitz inhibitors (50-52) Double-headed inhibitors with overlapping or non-overlapping binding sites are reported from plant sources (30,53).

The chemical modification of the functional groups of the inhibitor by selective reagents indicated that lysine residues in AATI are essential for the inhibitory activity against trypsin. This suggests that lysine is perhaps at active site of the inhibitor. The modification of arginine residues with 1, 2 cyclohexanedione did not have any effect on trypsin inhibitory activity. Trypsin inhibitors can be divided into two mechanistic classes 1) lysine type and 2) arginine type inhibitors. Protease inhibitors from the seeds of cowpea , *Inga laurina* and black gram have lysine in the active site (26,27,54) and those from, *Clitoria ternatea*, *Fagopyrum esculentum* seeds have arginine in the active site (55,56) . Serpins without arginine and lysine in the active centre are also reported from *Delonix regia* seeds (49).

CONCLUSION

Our results demonstrate that AATI a monomeric protein with a molecular mass of 20-kDa,low half cyctine content,ability to inhibit trypsin only, belongs to kunitz type inhibitors. Further studies on the interaction of AATI with mid gut proteases of insects are under progress.

ACKNOWLEDGEMENT

We are greateful to Senior Research Officer and National Vice President, Indian Dietetic Association, Food chemistry division National institute of nutrition (NIN), Hyderbad for getting the protein sample analysed for its composition of amino acids.

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